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Secretion of microvesicular miRNAs in cellular and organismal aging

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ABSTRACT

Changes of factors circulating in the systemic environment during human aging have been investigated for a long time. Only recently however, miRNAs have been found to be secreted into the systemic and tissue environments where they are protected from RNAses by either carrier proteins or by being packaged into microvesicles. These miRNAs are then taken up by recipient cells, changing the cellular behavior by the classical miRNA induced silencing of target mRNAs. The origin of circulating miRNAs, however, is in most instances unclear, but senescent cells emerge as a possible source of such secreted miRNAs. Since differences in the circulating miRNAs have been found in a variety of age-associated diseases, and accumulation of senescent cells in the elderly emerges as a possible detrimental factor in aging, it is well conceivable that these miRNAs might contribute to the functional decline observed during aging of organisms.
Therefore, we here give an overview on current knowledge on microvesicular secretion of miRNAs, changes of the systemic and tissue environments during aging of cells and organisms. Finally, we summarize current knowledge on miRNAs that are found to be specific for age-associated diseases.

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1. Introduction

Although much progress has been achieved in aging research over the past decades, the individual heterogeneity of the aging process in humans still remains puzzling and one of the major challenges for our societies. It is therefore mandatory to improve our understanding of the normal aging process, as aging is the substrate on which age-associated diseases will grow. Such a functional understanding on a molecular level might help to design strategies for prevention and therapy of the age-related losses in cell, tissue and organism functionalities.

Here we will summarize current knowledge on the secretion of miRNAs and their potential impact on cellular and organismal aging processes.

2. Cellular senescence, organismal aging, and the systemic environment

2.1. Cellular senescence

Senescent cells have been widely studied as a model system of aging ever since the replicative limit of normal human somatic cells in culture had been described by Hayflick more than four decades ago (Hayflick, 1965). Senescent cells are characterized by a combination of changes in cell behavior, structure and functions including an irreversible growth arrest, resistance to apoptosis and alteration in gene expression (Campisi and d'Adda di Fagagna, 2007). Moreover, a senescent phenotype is also induced by various physico-chemical stressors that induce DNA damage and chromatin disruption as well as by oncogenic signals (Cabrera et al., 1992; Maruyama et al., 2009). Since senescent cells never re-enter the cell cycle, cellular senescence is suggested to prevent malignant transformation of potentially mutated cells and thus contributes to tumor suppression. In contrast, senescent cells persist within tissues and are not eliminated by apoptosis, and their altered functional profile alters tissue microenvironments in ways that can promote both cancer and aging phenotypes (Krtolica and Campisi, 2002; Rodier et al., 2007). Recently it was shown that premalignant mammary epithelial cells exposed to senescent human fibroblasts in mice irreversibly lose differentiated properties, become invasive and undergo full malignant transformation (Parrinello et al., 2005).

Abbreviations: ESCRT, endosomal sorting complex required for transport; ILV, intraluminal vesicles; miRNA, microRNA; mRNA, messenger RNA; MVB, multivesicular bodies; MSC, mesenchymal stem cell; PM, plasma membrane; Rab, Ras-related in brain; RISC, RNA-induced silencing complex; rRNA, ribosomal RNA; SASP, senescence-associated secretory phenotype.

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2.2. Cellular senescence in vivo

By now, the presence and age-related accumulation of senescent cells in vivo have become well accepted (Campisi and d'Adda di Fagagna, 2007; Campisi and Sedivy, 2009) and it is well established that senescent cells in vivo contribute to age-associated diseases like atherosclerosis (Erusalimsky, 2009; Erusalimsky and Skene, 2009; Minamino and Komuro, 2007). Moreover it was shown that cellular senescence limits the extent of fibrosis following liver damage and underscores the interplay between senescent cells and the tissue microenvironment (Krizhanovsky et al., 2008).

In spite of these variously good and bad effects of cellular senescence, recent studies support the idea that accumulation of senescent cells with the aging of organisms accelerates age-associated diseases and loss of tissue function (Baker et al., 2011). In addition, the reactivation of telomerase in mice delays the onset of loss of tissue functionality (Jaskelioff et al., 2011), and even increases mouse life span (Bernardes de Jesus et al., 2012).

2.3. Senescent cells and their microenvironment

Besides the altered functionality of the cells themselves, detrimental effects of senescent cells might be due to an altered secretion phenotype. Indeed, senescent cells develop a senescence-associated secretory phenotype (SASP), where cytokines, extracellular matrix proteins and proteases, as well as other factors that alter the behavior of neighboring cells have been found. The senescent secretome of fibroblasts has been well established by the identification of various secreted factors that contribute to senescence like insulin-like growth factor binding protein 7 (IGFBP7) (Wajapeyee et al., 2008), interleukin-8 (IL-8) receptor binding chemokines (Acosta et al., 2008a, 2008b), IL-6 (Kuilman and Peeper, 2009), but also key components of the Wnt pathway, insulin-like growth factor 1 (IGF1), transforming growth factor-β (TGFβ), and plasmin (Kuilman and Peeper, 2009). The SASP includes inflammatory cytokines that are thought to drive aging and age-related disease (Finch and Crimmins, 2004). Indeed, some SASP factors, when chronically present, can disrupt tissue structure and differentiation (Parrinello et al., 2005), and others can promote malignant phenotypes in nearby pre-malignant cells (Bavik et al., 2006; Coppé et al., 2008; Krtolica et al., 2001; Liu and Hornsby, 2007). On the other hand, some SASP factors may be beneficial. For example, some reinforce the senescence growth arrest in an autocrine manner (Acosta et al., 2008b; Kuilman and Peeper, 2009; Kuilman et al., 2008). Others may allow damaged cells to communicate their compromised state (Rodier et al., 2009) in order to stimulate tissue repair or limit pathology (Krizhanovsky et al., 2008).

2.4. Senescent cells, the systemic environment and miRNAs

Accumulation of damage in cells and tissues has been accepted as one of the major driving forces of aging and age related diseases (Kirkwood, 2005). Several systemic factors have been found to change with age so far, among them chemokines like chemokine (C–C motif) ligand 11 (CCL11), whose levels correlate with neurogenesis (Villeda et al., 2011), and hormones like the growth hormone (Corpas et al., 1993) as well as the sulfated form of dehydroepiandrosterone (Baulieu, 1996).

In most tissues, there is an age-related decline in stem cell functionality but not a depletion of stem cells. Their ability to self-renew and differentiate is essential for homeostasis and regeneration of tissue and organs. The functionality of these cells declines with age (Rando, 2006). One factor that contributes to this functional decline is the systemic environment of old organisms (Conboy et al., 2005). This decline in functionality might be either due to factors that actively inhibit successful tissue regeneration or due to the absence of promoting factors in the elderly. In contrast, factors present in the systemic environment of young animals promote successful tissue regeneration (Matsumoto et al., 2009).

Such factors are still largely unknown, but some are slowly emerging. Among these are proteins, Wnt, TGF-β (Carlson et al., 2009) and IGF-1 signaling molecules (Mayack et al., 2010) that are suspected to be factors contributing to the functional decline. However, the source of such factors is currently unknown. It can be imagined that any other types of endocrine and inflammatory signaling molecules by their presence or absence might contribute to such an impairment of tissue functionality.

Recently, such "endocrine" functions have been attributed to miRNAs, as miRNAs are not only found intracellularly, but are also detectable outside of the cells, including various body fluids (e.g., serum, plasma, saliva, urine and milk) (Chen et al., 2012), where they circulate unimpeded by RNAses due to association with protective proteins or by being packaged into microvesicles (Viaud et al., 2008). But how are miRNAs generated, and how do they end up in microvesicles?

3. Microvesicular miRNAs

3.1. MicroRNA biogenesis and turnover

MiRNAs comprise a large family of ~21-nucleotide-long RNAs that have emerged as key post-transcriptional regulators of gene expression and have revolutionized our comprehension of the post-transcriptional regulation of gene expression. miRNAs are processed from primary transcripts (pri-miRNAs), which are either transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes. Pri-miRs are then processed to precursor miRNAs (pre-miRs) by the RNA endonucleases Drosha and Pasha and are exported to the cytoplasm where dicer cuts the pre-miRs into the mature miRNA duplexes. The mode of action in silencing depends on the recognition of target mRNAs by single stranded miRNAs that get incorporated into the RNA induced silencing complex (RISC). Binding may depend on the "seed" region consisting of nucleotides 2–8 of the miRNA only. One miRNA is able to regulate up to hundred mRNA targets and therefore seems to orchestrate a large variety of cellular processes similar to transcription factors (Lim et al., 2005; Stefani and Slack, 2008), but also in concert with transcription factors forming feed forward loops (Shalgi et al., 2007).

In contrast to miRNA biogenesis, turnover of miRNAs has received only limited attention to date. It is generally thought that miRNAs represent highly stable molecules and, experimentally using RNA polymerase II inhibitors or depletion of miRNA processing enzymes, have indicated that the half-lives of miRNAs in cell lines or in organs such as liver or heart correspond to many hours or even days (Gatfield et al., 2009; Großhans and Chatterjee, 2010; Krol et al., 2010; van Rooij et al., 2007).

miRNA stability is influenced by the 3′ end sequence motif or modification that mark miRNAs for degradation or protect them against exonucleolytic activity, depending on the specific miRNAs and the tissues. In liver cells, a single adenine residue added to the 3′ end of miR-122 prevents trimming and protects the miRNA against exonucleolytic degradation (Katoh et al., 2009).

3.2. Microvesicles

It was long believed that the way of communication between cells and tissues depends to a large extent on protein-based signaling systems exemplified by soluble secreted factors like cytokines, chemokines, neurotransmitter, enzymes and hormones. In principle there were two classes of signaling systems described. One system depends on direct cell–cell contact (also known as juxtacrine

signaling) exemplified by the formation of gap junctions, intercellular bridges or synaptic connections. On the other hand a second contact independent system has been described. In the latter case soluble factors are either secreted into the intercellular space where they are able to effect nearby cells (also known as paracrine signaling) or into the circulation where they can cover long distances and affect faraway recipient cells (also known as endocrine signaling).

With the recent discovery of microvesicles a new mechanism of cell communication participating in the contact independent system was proposed. Microvesicles are vesicles which bud from a cellular membrane and contain, depending on their origin, proteins, mRNAs, miRNAs and/or DNA (Mathivanan et al., 2010a). In contrast to the abovementioned and well understood protein-based signaling systems, microvesicles have the advantage that they deliver not only one but multiple (potentially synergistic) messages, whereby they might be able to influence and change the cellular behavior at several stages of protein expression allowing rapid control over targeted cells.

Microvesicles were found to be produced by various cells e.g. by dentritic cells, mast cells, B-cells, T-cells, platelets, but also in neurons, oligodentrocytes, epithelial cells, endothelial cells, embryonic fibroplast cells, microglia, neuroglial cells and several tumor cell lines to name but a few (Hu et al., 2012; Mathivanan et al., 2010a). Furthermore they were also found in several body fluids such as serum, plasma, urine, ocular fluids, amniotic fluid, ascites, broncheoalveolar lavage, cerebrospinal fluid, seminal plasma, breast milk, tears and saliva (Cocucci et al., 2009; Hu et al., 2012; Mathivanan et al., 2010a; Pant et al., 2012; Zhu and Fan, 2011).

3.3. The Babylonian language problem

To date, three different types of microvesicles seem to crystallize out of a plethora of names (Table 1): ectosomes, exosomes and apoptotic blebs. In principle they are distinguished by their size, shape, density, origin as well as protein membrane composition (Mathivanan et al., 2010a; Thery et al., 2009).

However, no commonly accepted nomenclature for microvesicles has yet been established, resulting in a confusing and confounding terminology. For example several names such as microparticles, microvesicles, nanoparticles, shedding microvesicles, ectosomes, exosomes, exosome-like vesicles, dexosomes, texosomes, oncosomes, apoptotic blebs, apoptotic bodies, to name a few were used for a mixed population of microvesicles. Only recently, we have begun to understand that in body fluids different species of vesicles exist and that we need to isolate them based on differences in composition, size or shape as done in a couple of recent reports (Mathivanan et al., 2010b). Therefore, a close look on the performed isolation method of vesicles is highly recommended when reading literature and a commonly agreed terminology would be highly welcome.

Table 1
Comparison of microvesicle types.

	Exosomes	Ectosomes	Apoptotic bodies
Size [nm]	30–100 nm	100–1000 nm	50–5000 nm
Floating density [g/ml]	1.10–1.21	N.K.	1.16–1.28
Sedimentation [g]	100,000–110,000	10,000–100,000	1500–100,000
Cargo	mRNA, miRNA, proteins	mRNA, miRNA, proteins	mRNA, miRNA, rRNA, DNA, proteins
Origin	Late endosomes	Plama membrane	Apoptotic cells
Specific marker	Tetraspanins(CD81, CD63) ESCRT member (Alix, TSG 101)	Integrins, selectins, metalloproteinases	Histones, DNA
Morphology	Cup shaped	Irregularly	Irregularly
Uptake of cargo	Specific	Specific	N.K.
Mode of release	Fusion of MVB with PM	PM blebbing by actomyosin contraction	PM blebbing by actomyosin contraction

4. Biogenesis of microvesicles

4.1. Exosomes

Exosomes are small cup shaped membrane vesicles of 30–100 nm in diameter which originate from the endosomal compartment (Simpson et al., 2008, 2009). The name goes back to 1983, when Pan and Johnstone described microvesicles responsible for externalizing receptors during erythrocyte maturation, which are formed by inward budding of vesicles into the late endosomal compartment (Pan and Johnstone, 1983). Such inward budding of the late endosomal limiting membrane, is by now known to encapsulate cytosolic 'cargo' into intraluminal vesicles (ILV) whereby large multivesicular bodies (MVB) are formed (van Niel et al., 2006). MVBs enriched in ILV can either fuse with lysosomes, if their content is intended for degradation or with the plasma membrane whereby ILVs are released into the extracellular space as "exosomes" (Simpson et al., 2009).

Although it is still unknown how the formation of MVBs works on a molecular basis, mechanisms have been proposed.

The first requires the ESCRT (endosomal sorting complex required for transport) machinery to comprise mainly mono ubiquitinated cargo for lysosomal degradation (Babst, 2005). The formation of the second type of MVBs is based on an alternative mechanism involving lipid rafts enriched in sphingolipid ceramide (Trajkovic et al., 2008). The third mechanism was proposed by Rana et al. assuming that proteins are incorporated by special membrane domains resistant to detergents because of their lipid composition and enriched in tetraspanin (Rana and Zöller, 2011).

Whichever mechanism or combination of mechanisms will be operative, several necessary components for exosome formation have by now been identified, e.g., Ras-related in brain 27a (Rab27a) which influences the size of MVBs (Ostrowski et al., 2010), Rab27b which is controlling the direction of MVBs (Ostrowski et al., 2010), Rab35 which contributes to the docking of exosomes to the plasma membrane (Hsu et al., 2010), hepatocyte growth factor receptor substrate (Hrs) which is necessary for ILV formation (Razi and Futter, 2006), soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNAREs) having a role in the fusion process of MVBs and the cellular plasma membrane (Bobrie et al., 2011) and myosins (Pant et al., 2012).

4.2. Ectosomes

Stein and Luzio defined ectosomes as right-side-out-orientated vesicles containing cytosolic components which are released from the surface of polymorphonuclear leucocytes attacked by a complement (Stein and Luzio, 1991). Today the term "ectosomes" is used in a more general way and defined as microvesicles which directly bud from the cellular plasma membrane. Ectosomes are of irregular shape and vary in size between 100 and 1000 μm (Mathivanan et al., 2010a). In contrast to the plasma membrane that they originate from, they expose phospholipid phospatidylserine (PS) on their surface (Zwaal and Schroit, 1997). Furthermore they contain metalloproteinases, which were shown to influence the extracellular space thereby promoting tumor metastasis and invasion, selectins and integrins (Coppé et al., 2008; Mathivanan et al., 2010a).

Ectosomes are continuously produced by many if not all cells in vitro but the stimulating agents, which are able to enhance their production, vary between the different cell types (Sadallah et al., 2011). In addition they contain proteins, mRNAs and miRNAs. The

composition depends on cell type, cellular state, and stimulating agent (Cocucci and Meldolesi, 2011).

For the shedding of ectosomes a local disassembly of the cytoskeleton as well as the vesicle abscission process is necessary (Cocucci and Meldolesi, 2011). Therefore increasing cytosolic Ca^{2+} concentrations, activated p38 MAPK Curtis et al. (2009), acid sphingomyelinase (Bianco et al., 2009) and the Rho–ROCK axis (Pinner and Sahai, 2008) as well as the regulative enzymes calpain, a calcium-dependent cytosolic protease (Miyoshi et al., 1996), flippase, floppase, scramblase and gelosin were shown to be involved (Enjeti et al., 2008).

4.3. Apoptotic blebs

Apoptotic blebs are released by cells during the late stage of apoptosis through pinching off from the cellular plasma membrane (Beyer and Pisetsky, 2010). The abscission of apoptotic blebs is performed through the intracellular increase of the hydrostatic pressure followed by the cellular contraction with the aid of actomyosin (Charras et al., 2005, 2008). They are greater than 50 nm in diameter and of irregular shape (Mathivanan et al., 2010a). They contain proteins, mRNAs, miRNAs as well as DNA and expose phosphatidylserine (Théry et al., 2001).

5. Packaging of miRNAs into vesicles and releasing to the cellular environment

In 2007 Valadi and coworkers were the first to show that miRNAs, besides mRNAs and proteins, are packaged into microvesicles (Valadi et al., 2007). Surprisingly, the miRNA content of microvesicles does not necessarily correspond to the cytosolic repertoire of miRNAs, as the miRNA profile of hepatocellular cancer cells and their microvesicles does differ (Kogure et al., 2011). Only 134 of 424 cellular detectable miRNAs were also found in the exosomes of hepatocellular cancer cells. Of these 134 vesicular miRNAs, 25 were higher and 30 miRNAs were less expressed compared to the intracellular miRNA expression levels. Interestingly, 11 miRNAs were solely identified in microvesicles, strongly suggesting that a specific and controlled packaging mechanism needs to exist (Kogure et al., 2011).

So far, only the first indications on this mechanism are known from studies using human monocytes, where mRNAs, miRNAs and components of the RNA-induced silencing complexes (RISCs) are concentrated in GW-bodies, which are distinct foci within the cytoplasm where many necessary proteins for miRNA gene silencing accumulate. These GW-bodies accumulate at the membrane of late endosomes and MVBs which might facilitate the loading of RNA into exosomes thereby contributing to intercellular transport or post-transcriptional control of intracellular miRNA levels (Gibbings and Voinnet, 2010). Although all components of RISC were found to be localized at the membranes of late endosomes and MVBs, only trinucleotide repeat containing 6 (TNRC6), pre-miRNAs, fragments of pre-miRNA stem loops and miRNAs were found to be enriched within exosomes (Chen et al., 2010; Gibbings et al., 2009). It is known that TNRC6 directly binds to Argonaute (Ago) thereby initiating the mRNA decay (Chen et al., 2009). A mutant of TNRC6 lacking, in its C-terminus, caused a reduced miRNA activity and the accumulation of miRISC (AGO, miRNA, mRNA and TNRC6) indicating that its C-terminus might be necessary for the dissociation of miRISC (Zekri et al., 2009). Poly(A) binding protein (PABP) which potentially binds at the C-terminus of TNRC6 was shown to bind directly to tumor susceptibility gene 101 (TSG101) thereby providing the link between the RISC complex and exosomes (Schlundt et al., 2009).

Interestingly similar inhibiting effects on miRISC dissociation and reduced levels of exosomal TNRC6 were observed when ESCRT was knocked out, suggesting that ESCRT is necessary for sorting TNRC6 into exosomes (Gibbings et al., 2009). In addition ESCRT II might have a role in sorting miRNA into exosomes since it is able to bind RNA directly (Irion and St Johnston, 2007). Summarizing functional links between miRNAs and the ESCRT machinery, where RISC as well as the ESCRT complex might work together in a common pathway to load miRNAs into exosomes were found.

In contrast, neutral sphingomyelinase 2 (nSMase2), which is regulating the biosynthesis of ceramide, but not the ESCRT machinery is necessary for the production of exosomes and their enclosed miRNAs in human T cells (Mittelbrunn et al., 2011). This indicates that the uptake of miRNAs by the aid of the ESCRT machinery may not be the only miRNA loading mechanism and might depend on the cell type and condition.

Packaging of miRNAs into ectosomes is currently even less understood. It is assumed that ectosomes shed from plasma membrane regions enriched in lipid rafts and that proteins, mRNAs and miRNAs supposed to be packaged into ectosomes, accumulate at these regions (Cocucci and Meldolesi, 2011). However, there are several independent studies indicating that ectosomes mainly contain mRNAs and show lower levels of miRNAs compared to exosomes. Skog et al. determined in 2008 by bioanalyzer that ectosomes originating from glioblastoma cells predominantly contain mRNAs and barely miRNAs (Skog et al., 2008).

In any case, packaging of miRNAs into vesicles is a key step for protecting the miRNAs against the ubiquitous RNAses in the extracellular space, clearly necessary for the stability of extracellular miRNAs, which are reportedly more resistant against extreme temperatures, extreme pHs, prolonged storage and freeze–thaw cycles (Chen et al., 2008; Mitchell et al., 2008).

6. Microvesicular miRNAs: uptake mechanisms by target cell

After packaging miRNAs into microvesicles, they can be transported through the interstitium or even the peripheral blood (Hunter et al., 2008). Interestingly microvesicular miRNAs were also found in serum (Skog et al., 2008), breast milk (Kosaka et al., 2010) and saliva (Michael et al., 2010). Recently it was shown that engineered microvesicles expressing a neuron specific peptide fused to the surface marker CD107b, are even able to cross the blood–brain barrier and to deliver siRNA to murine neurons, microglia and oligodendrocytes after their intravenous injection (Alvarez-Erviti et al., 2011).

The first RNAs to be proven as functionally delivered and translated by microvesicles were mRNAs originating from mouse mast cells. Their vesicular transport to human mast cells resulted in the expression of three mouse proteins in human cells (Valadi et al., 2007).

In terms of miRNAs, by now several independent studies demonstrate, that functional microvesicular miRNAs are taken up by targeted cells in a sufficient amount to repress the translation of target genes. For example in 2010 the group of Pegtel observed a microvesicular transport of miR-150 from THP-1 to HMEC-1 cells and the consequent silencing of its target c-Myb in the recipient cells (Zhang et al., 2010).

These results also make clear that our current transfection procedures might work so well since they just mimic the natural transfer of RNA and DNA species via microvesicles. But how are the microvesicular miRNAs then taken up by the target cells? There are two mechanisms that have been observed so far.

6.1. Plasma membrane fusion

Exosomes can be transferred between dendritic cells (DC) and are taken up by membrane fusion. Through the expression of a GFP-tagged exosomal surface marker protein by the cells of origin, the recipient plasma membrane exhibited fluorescing GFP patterns after exosomal uptake. This is taken as a proof that vesicles are taken up by membrane fusion (Montecalvo et al., 2012).

6.2. Endocytosis

By live-cell microscopy Tian et al. observed that microvesicles from rat PC-12 cells can be efficiently taken up by endocytosis and consequently transferred to the perinuclear region probably mediated by the cytoskeleton (Tian et al., 2010). Similarly, we recently observed a reduced uptake of microvesicles originating from endothelial cells by human mesenchymal stem cells (MSCs), when endocytosis of MSCs was blocked by the overexpression of a dominant negative dynamin construct (K44A) (Schraml et al., unpublished data).

7. Functional consequences of microvesicular miRNA uptake

Several functions of microvesicles were demonstrated in recent years including effects on cell growth, proliferation, development, differentiation and cell death as well as on coagulation, the immunological processes, viral infections, prion infections and cancer progression (Janowska-Wieczorek et al., 2005; Vlassov et al., 2012; Zhu and Fan, 2011). By now, several examples exist showing that the transfer of miRNAs to target cells results in changes of the target cell behavior strongly indicating that microvesicular miRNAs do indeed make part of the cell to cell communication system. Such examples come mainly from studies in the cancer field, where microvesicles isolated from the highly metastatic gastric cancer cell line AZ-P7a secrete increased levels of the let-7 miRNA family compared to low metastatic AZ-521. Let-7 miRNAs are known to function in tumor suppression since they target the oncogenes RAS and HMGA2. It is assumed, that metastatic AZ-P7a cells release let-7 miRNAs via microvesicles in order to get rid of this tumor suppressive miRNAs and to maintain their own tumorigenesis (Ohshima et al., 2010).

Similarly, several examples in the immune system exist. Unidirectional transfer of miRNA within CD63 positive exosomes, derived from T cells to antigen-presenting cells was shown (Mittelbrunn et al., 2011). Furthermore miRNAs circulating in human breast milk might support the development of the infants' immune system (Kosaka et al., 2010) and miRNA transfer by microvesicles seems also important in viral infection or defense (Pegtel et al., 2010).

8. Aging, senescence and microvesicular miRNAs

Recently, it has been reported that in both replicative and cell damage-driven senescence, cells increase overall secretion, termed SASP, which is characterized by the secretion of a wide variety of factors, including peptide hormones, as well as the release of microvesicles (Acosta et al., 2008a, 2008b; Campisi, 2008; Campisi and d'Adda di Fagagna, 2007). Of note, this secretory activity and microvesicle formation in senescent cells is also regulated by p53 (Yu et al., 2006).

It is known that miRNAs have some influence on the aging process (Gorospe and Abdelmohsen, 2011; Hackl et al., 2010), but little is known in regard to the systemic environment. So far, several studies have compared samples from young versus healthy elderly individuals of different species from worm to human tissues in regard to the intra-cellular miRNA expression profile. Indeed, different expression levels of several miRNAs have been found with age, e.g., the miR-17-92 cluster (Hackl et al., 2010) let-7 (Peng et al., 2012) and miR-34a (Li et al., 2011). Upregulation of specific miRNAs seems to induce senescence in cells, among them miR-34a in human cells (Christoffersen et al., 2010), miR-203 in melanoma cells (Noguchi et al., 2012) and miR-101, miR-137 and miR-668 in keratinocytes (Shin et al., 2011).

While not much has been published on miRNA secretion during the process of aging, it becomes increasingly clear that miRNAs might be biomarkers of several age-associated diseases. Since several of these diseases seem to be connected to the increase of senescent cells, we summarize in the following the current knowledge on miRNAs that have been found in the serum or plasma of such patients (Fig. 1).

9. Microvesicular miRNAs in age-associated diseases

The promises of miRNAs as diagnostics and therapeutics are very high even if we are only at the beginning of developing this RNA species into biomedical tools. Still, we are convinced that the need for personalized medicine will boost the search and identification of single miRNAs and/or miRNA signatures characterizing specific patients and thus will help in guiding therapies. This seems an urgent need considering that 90% of the available drugs are efficient only in 40% of the patients.

9.1. Cardiovascular disease

Cardiovascular diseases are the primary cause of death and cause for many conditions that severely impact on the quality of life at old age. Endothelial cells seem to counteract this as they secrete microvesicles, containing miR-143 and miR-145 that are taken up by VSMC preventing VSMC de-differentiation (Hergenreider et al., 2012). Injection of vesicles containing both miRNAs into ApoE$^{-/-}$ mice then indeed resulted in reduced atherosclerotic lesion formation (Hergenreider et al., 2012). In addition, it was found that deregulation of both miRNAs contributes to aberrant VSMC plasticity occurring during vascular diseases (Jakob and Landmesser, 2012).

During atherosclerosis, miR-126 has been found to be secreted via apoptotic blebs by endothelial cells. This in turn enhances the production of the anti-inflammatory chemokine CXCL12 as well as its receptor CXCR4 and promotes the recruitment of endothelial progenitor cells supposedly as a protective mechanism, where the progenitor cells help to maintain tissue homeostasis (Zernecke et al., 2009). The protective role of miR-126 was demonstrated also in vivo when apoptotic blebs enriched in miR-126 originating from human umbilical vein endothelial cells (HUVECs) were injected into high fat diet ApoE$^{-/-}$ mice, resulting in a reduced lesion size and macrophage accumulation through CXCR4 expression (Zernecke et al., 2005).

Counterintuitively, downregulation of miR-126 in the plasma of patients suffering from coronary artery disease has been reported (Fichtlscherer et al., 2011). It might be speculated, that the targeted transport of vesicular miRNAs to the sites of atherosclerotic lesions could cause a reduced detection of freely circulating miRNA levels (Zhu and Fan, 2011).

Moreover microvesicles also protect against progression of chronic kidney damage by inhibiting capillary refraction, glomerulosclerosis, and tubulointerstitial fibrosis by delivering pro-angiogenic miR-126 and miR-296 (Cantaluppi et al., 2012).

In any case, these studies taken together indicate that endothelially derived miRNA carrying microvesicles might contribute to the progression of atherosclerosis.

9.2. Neurodegenerative diseases

Alzheimer's disease is the most frequent neurodegenerative disease in humans. It is characterized by the incident of amyloid plaques, which are insoluble extracellular depositions containing amyloid beta peptide (Aβ) (Cai et al., 1993). In Alzheimer's patients, typical exosomal markers were found to be located at sites of amyloid plaques (Rajendran et al., 2006), giving a first indication that microvesicles might be involved in neurodegenerative diseases. Indeed, microvesicles have been shown to be secreted by several neuronal cell types, thereby contributing to the physiology and synaptic plasticity of the central nervous system (Bellingham et al., 2012). It is also of note, that microvesicles have been found to be able to pass the blood–brain barrier and to deliver functional siRNA

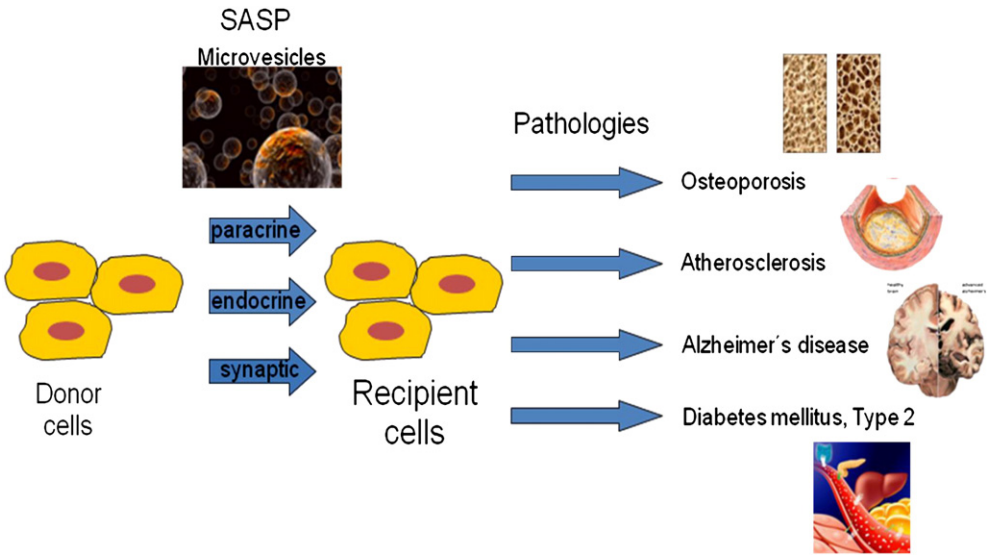

Fig. 1. Senescent donor cells contribute to the senescence-associated secretory phenotype (SASP) by secreting not only soluble proteins but also microvesicles either in a paracrine, endocrine or synaptic manner. These vesicles are taken up by recipient cells and may cause or contribute to age related pathologies like osteoporosis, atherosclerosis, Alzheimer's disease or diabetes mellitus, type 2.

to neurons (Alvarez-Erviti et al., 2011), which opens up the possibility of cross talk via this usually very tight and selective barrier.

Furthermore, microvesicles from Alzheimer's patients have been found to be enriched in Aβ, its precursor protein APP and some components of γ-secretase and might thereby contribute to the spread of Alzheimer's disease (Vella et al., 2008). Surprisingly, microvesicles isolated from the cerebrospinal fluid of diseased patients contain around 60 differentially expressed miRNAs compared to healthy controls (Cogswell et al., 2008). So far it is unclear, if these miRNAs might in consequence also be involved in the pathogenic mechanisms of Alzheimer's disease, but the findings again support the importance of microvesicular miRNAs which might serve at least as biomarkers of diseases like Alzheimer's disease.

9.3. Diabetes mellitus, type 2 (T2DM)

The number of patients suffering from T2DM is dramatically increasing in developed and developing countries (Zimmet et al., 2001).

First indications of deregulated microvesicular miRNAs in plasma of patients suffering from T2DM compared to healthy age and sex matched controls are available and provide a potential diagnostic signature containing 5 miRNAs, including reduced miR-126 level (Zampetaki et al., 2010).

Interestingly, it was already demonstrated that increased miR-126 level promote vascular endothelial growth factor (VEGF) signaling and has a positive effect on vascular protection (Zernecke et al., 2009). It is known that the monocytes of patients suffering from T2DM exhibit reduced VEGF sensitivity which is contributing to an impaired collateral vessel development. This effect might be due to a reduced microvesicular transport of miR-126 to monocytes (Zampetaki et al., 2010).

9.4. Osteoporosis

So far, no study has addressed secreted miRNA in bone disorders of the elderly, however, it is conceivable that miRNAs will be associated with this disease as well, since several miRNAs are known to be regulated during osteogenic differentiation (reviewed by Schraml and Grillari, 2012).

Recently our group could show that microvesicles originating from senescent endothelial cells are taken up by MSCs thereby inhibiting osteogenesis via miR-31 delivery Weilner, Schraml and Grillari, unpublished observations. Additionally, circulating miR-31 levels were also found to be significantly increased in plasma of donors older than 55 as well as in patients suffering from osteopenia compared to donors younger than 25 years Weilner, Schraml and Grillari, unpublished observations.

These results might provide a link between the age associated incidence of reduced bone healing or osteopenia and accumulating senescent endothelial cells with age in vivo.

10. Concluding remarks

The still quite recent identification of secreted and/or microvesicular miRNAs in the systemic environment, in interstitial fluids or other body fluids poses a formidable tool to identify and use miRNAs as diagnostic signatures. Developing such signatures is of high concern as personalized medication and therapy depends on “personalized” diagnostic tools. The here presented examples of miRNAs that are associated or sometimes even causally linked to age-associated diseases might provide such a toolbox of biomarkers. As small non-coding RNAs have been used in clinical trials by now, miRNAs will certainly also find applications as therapeutic targets. Especially for those miRNAs that circulate in the systemic environment, drug targeting might be comparably easy due to good accessibility in the blood.

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